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Exon array analysis reveals genetic heterogeneity in atypical femoral fractures. A pilot study

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Abstract Atraumatic subtrochanteric and diaphyseal (atypical) femoral fractures are a rare, but important adverse event in patients treated with potent anti-resortive agents. The mechanisms involved are unknown and particularly the association with genetic variants has not been explored. The aim of the study was to identify rare genetic variants that could be associated with the occurrence of these fractures. We performed a genome-wide analysis of up to 300,000 variants, mainly distributed in gene coding regions, in 13 patients with atypical femoral fractures and 268 control women, either healthy or with osteoporosis. Twenty one loci were more frequent in the fracture group, with a nominal p value between 1×10^{-6} and 2.5×10^{-3}. Most patients accumulated two or more allelic variants, and consequently the number of risk variants was markedly different between patients and controls ($p = 2.6 \times 10^{-22}$). The results of this pilot study suggest that these fractures are polygenic and are associated with the accumulation of changes in the coding regions of several genes.

Keywords Atypical fractures · Polymorphisms · Genetic association study · Osteoporosis · Hedgehog · Rare variants

Introduction

Several anti-resorptive drugs, including aminobisphosphonates, denosumab, and strontium ranelate, and the bone anabolic agent teriparatide, have been shown to decrease the incidence of both vertebral and peripheral fractures [1]. These drugs not only have a beneficial effect, increasing bone mineral density and decreasing the risk of fractures, but they also have a good safety profile and are usually well tolerated. However, some patients on anti-resorptive agents may develop certain distressing side effects, such as osteonecrosis of the jaw or atypical femoral fractures [2, 3].

Although these complications are fortunately infrequent, they cause significant concern and may pose important treatment difficulties. Atypical fractures are atraumatic or low-trauma fractures located in the subtrochanteric region or the femoral shaft, with transverse or short oblique configuration [4]. Despite some controversial results [5], most studies have found an association between anti-resorptive drugs, particularly aminobisphosphonates, and the occurrence of atypical fractures [6]. The incidence in patients on long-term bisphosphonate therapy has been estimated to be about 0.1–0.2 % [4]. The pathogenesis has not been elucidated [7]. Nevertheless, their rare occurrence suggests that the individual predisposition plays an important role. In fact, patients suffering bilateral fractures are not uncommon and represent about 25 % cases in some series [4]. Different from osteoporosis, which is quite common in

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the population, these fractures only affect to a minor proportion of individuals. Therefore, we hypothesized that a rare genetic variant might drive the individual susceptibility. To explore this hypothesis, we analyzed exome genetic variants in a group of patients with atypical fractures and compared the results with those in control individuals and patients with osteoporosis without atypical fractures.

Materials and methods

Subjects

We studied 13 women with atypical femoral fractures according to the ASBMR criteria [4]. Mean age was 77 years (range 57–87); 12 had been treated with bisphosphonates for 1–10 years (Table 1). Two had bilateral atypical fractures. The comparison group ($n = 268$) included 87 control women and 181 women of similar age with postmenopausal osteoporosis recruited from our osteoporosis clinic. Patients and controls provided informed consent and the study has been approved by the IRB.

Genotyping

DNA was extracted from the peripheral blood by standard commercial methods and analyzed with the Axiom 2.0 exome genotyping array in the Affymetrix GeneTitan® Multi-Channel (MC) Instrument. Cel files generated by the GeneTitan MC Instrument were automatically processed using the Axiom® Genotyping Algorithm version 1 (Axiom GT1), available through Genotyping Console™ v4.1. The Axiom® Exome Genotyping Array contains about 300,000 coding SNPs—including non-synonymous and synonymous SNPs as well as variants in splice and stop codons—and approximately 30,000 indels, among other variants.

Table 1 Patients with atypical femoral fractures

Age	Bilateral fractures	Anti-resorptive drug	Duration (months)
76	Yes	Alendronate	60
87	No	Risedronate	48
57	No	Alendronate	96
84	Yes	Alendronate	46
84	No	Alendronate	44
76	No	Alendronate	120
80	No	Risedronate	10
83	No	–	–
72	No	Alendronate	65
87	No	Risedronate	16
62	No	Ibandronate	30
78	No	Alendronate	48
83	No	Alendronate	58

Quality control and data analysis

In a replicated sample, the concordance rate of allele calling was 99.89 % prior to any filtering. Variants with calling rates <99.5 % and individuals with successful genotyping <95 % were excluded from the analysis. Likewise, markers with genotypes not following the Hardy–Weinberg equilibrium ($p < 0.0001$) were also excluded. A minor allele frequency threshold of 0.003 was set, thus assuring that each allele was found at least twice in the whole study population and avoiding spurious singletons due to mistaken allele calls. Finally, cluster graphs of markers with significant results were visually inspected to check that allele clusters were adequately separated.

As stated in Introduction, given the low frequency of atypical fractures, we hypothesized that they could be associated with alleles uncommon in the general population. Therefore, as frequently done in other studies, the analysis was restricted to rare variants (MAF < 0.03 in the control and patient groups combined). An adaptive permutation test was used to compare the allelic frequencies in both groups, computed with PLINK software v1.07 [8]. Odds ratios (OR) and the 95 % confidence intervals were calculated with the Cornfield method. In order to avoid divisions by zero while estimating OR, a conservative approach was used and 4 events were considered. Thus, when the frequency of a given allele was 0 in the control population, it was given a value 0.01 to compute OR. This value corresponds approximately to the rounded upper confidence limit of a 0 value, estimated as previously suggested ($4/532 = 0.007$) [9]. Pathway analysis was performed with DAVID software (http://david.abcc.ncifcrf.gov). Location and functional annotations were obtained from the Affymetrix website (http://www.affymetrix.com) and the PROVEAN web service available at the Craig Venter Institute (http://provean.jcvi.org/index.php) [10]. This software takes into consideration all transcripts and not only the canonical ones. The functional consequences of the changes in protein sequences were estimated with the SIFT prediction tool [11], also through the PROVEAN web site.

Results

We found 94,314 non-monomorphic loci, among the 295,988 markers successfully genotyped. The genotyping rate was 98.82 %. After filtering by the allele calling rate,

Hardy–Weinberg equilibrium and minor allele frequency, 25,046 markers were analyzed further. They included 8829 missense variants.

The 21 markers with the lowest p values (i.e., those below the arbitrary threshold of $p < 0.0025$) are shown in Table 2. Given the limited number of cases, only the most significant marker remained statistically significant after multiple-test adjustment (FDR 0.0007). They included 19 single nucleotide variants and 2 indels. There were 10 missense variants according to canonical annotations and 16 when alternative gene transcripts were included (Table 3). Given the MAF threshold set, the frequency of all variants was low in the control group. They also were relatively uncommon in the cases group, with allelic frequencies between 7 and 18 %. The estimated ORs varied between 7 and 70 (Table 2). Pathways analysis using the DAVID webtool (http://david.abcc.ncifcrf.gov/) did not reveal any pathway over-representation statistically significant after multiple test correction.

The number of risk variants present in each individual is summarized in Fig. 1. Several rare variants tended to accumulate in patients with fractures. Thus, one case had 2 variants, and 12 had 3 or more. On the other hand, 42 controls (15.7 %) had 1 rare variant, but none accumulated more than 1 (Fig. 1a). Thus, the difference in the overall distribution of the number of rare variants between cases and controls was highly significant (Fisher test p value 1.2×10^{-22}). Two patients had bilateral fractures; each carried 5 risk alleles. When the analysis was restricted to those variants likely to have a damaging effect on protein function according to SIFT software, a similar different distribution between cases and controls was found (Fig. 1b, $p = 1.4 \times 10^{-14}$).

Discussion

Atypical femoral fractures are rare, but they may have a strong impact on the quality of life and life expectancy of osteoporotic patients [12]. Therefore, there is great interest in identifying the pathogenetic mechanisms involved and which patients are at risk. Some clinical factors have been postulated, but the results reported are controversial. As with other bone-related disorders, including usual osteoporotic fractures [13], a genetically determined individual susceptibility has been suggested. In fact, some unique

Table 2 Variants with a trend for association with fractures (unadjusted $p < 0.0025$). The marker name corresponds to the Affymetrix probe designation

CHR	SNP	dbSNP	Alelle 1	Gene	Freq cases	Freq controls	Allele 2	p	OR
4	AX-83191953	rs140029551	T	PPEF2	0.192	0	C	1.0×10^{-6}	>21
2	AX-83175884	rs193151657	T	ACOXL	0.154	0.007	C	0.00022	24
17	AX-82887659	rs28641831	G	GGA3	0.154	0.007	A	0.00029	24
10	AX-11492492	rs41284088	T	LIPN	0.154	0.009	C	0.00039	19
5	AX-83184588	rs187220094	A	DOCK2	0.115	0.002	G	0.00039	70
10	AX-83008658	rs74741614	A	CCDC147	0.154	0.009	C	0.00044	19
11	AX-83019011	–	ACCACCCC	OR51T1	0.115	0.002	–	0.0005	70
14	AX-82932920	–	C	PCK2	0.115	0.004	–	0.00093	35
22	AX-40861967	rs16986560	T	CRYBB2	0.115	0.006	G	0.00152	23
2	AX-83196592	rs150632398	T	CXCR7	0.077	0	C	0.00154	>7
15	AX-83089650	rs149888951	C	EDC3	0.077	0	T	0.00154	>7
16	AX-82930686	rs116924445	T	SF3B3	0.115	0.017	C	0.00166	7
12	AX-30598385	rs61915927	C	SLC15A5	0.154	0.015	T	0.00175	12
9	AX-82908268	rs149329547	T	SLC2A6	0.077	0	C	0.00181	>7
17	AX-83232437	rs146173102	A	FOXK2	0.077	0	G	0.00204	>7
16	AX-83066135	rs147593839	A	CNGB1	0.077	0	G	0.00214	>7
2	AX-82923570	..	A	NAT8B	0.077	0	C	0.00214	>7
1	AX-82990102	rs149597734	T	HHAT	0.077	0	C	0.0022	>7
1	AX-16991963	rs113312394	C	OR2L13	0.077	0	G	0.00223	>7
11	AX-83121869	rs72947495	C	SYTL2	0.077	0	T	0.00243	>7
12	AX-83123433	rs139219535	C	SLC15A5	0.077	0	T	0.00246	>7

The frequencies of the minor allele (allele 1) in cases and controls are shown. To avoid divisions by 0 when estimating odds ratios (OR), the arbitrary value of 0.01 was given to the frequency in controls when the minor allele was not found in that group. p values from permutations

Table 3 Variants with a trend for association with fractures

SNP	Gene	Function Affy	Function CVI	Effects
AX-83191953	PPEF2	Missense	Missense	Tolerated
AX-83175884	ACOXL	Missense	Missense	Damaging
AX-82887659	GGA3	Synon	Synom	Tolerated
AX-11492492	LIPN	Intron	Missense	Damaging
AX-83184588	DOCK2	Intron	Missense	Tolerated
AX-83008658	CCDC147	Missense	Missense	Damaging
AX-83019011	OR51T1	–	Frameshift	–
AX-82932920	PCK2	–	–	–
AX-40861967	CRYBB2	Missense	Missense	Tolerated
AX-83196592	CXCR7	Missense	Missense	Damaging
AX-83089650	EDC3	Missense	Missense	Tolerated
AX-82930686	SF3B3	Intron	Missense	Tolerated
AX-30598385	SLC15A5	Synon	Synom	Tolerated
AX-82908268	SLC2A6	Missense	Missense	Damaging
AX-83232437	FOXK2	Intron	Missense	Tolerated
AX-83066135	CNGB1	Missense	Missense	Damaging
AX-82923570	NAT8B	–	–	–
AX-82990102	HHAT	Missense	Missense	Damaging
AX-16991963	OR2L13	Intron	Missense	Tolerated
AX-83121869	SYTL2	Intron	Missense	Damaging
AX-83123433	SLC15A5	Missense	Missense	Damaging

The SNP name corresponds to the Affymetrix probe designation. Functional annotations according to the Affymetrix database (Affy) and the PROVEAN web service at the Craig Venter Institute (CVI). Last column shows the functional effects of protein sequence changes according to the SIFT algorithm, run on the CVI website

patients with hypophosphatasia and atypical fractures have been identified, thus suggesting that genetic defects of the gene encoding alkaline phosphatase may predispose to atypical fractures [14]. However, most patients with this type of fracture have normal levels of alkaline phosphatase.

The aim of this study was to perform a genome-wide analysis searching for variants in coding regions predisposing to atypical fractures. We indeed found several variants that tended to be associated with this type of fracture, but, likely due to the small sample size, most of them did not survive the multiple test-adjusted threshold for significance. In order to identify variants actually associated with atypical fractures (not with osteoporosis), the comparison group included both healthy women and women with osteoporosis not suffering atypical fractures. Given the reported risk for genomic inflation in rare variant analyses [15, 16], instead of using a gene-based approach (i.e., burden tests, SKAT), we performed an analysis of single variants. By doing so, our results pointed to 20 genes associated with fractures and suggest that the underlying genetic architecture of these fractures is complex (i.e., several genes are involved). First, fractures do not appear to be the consequence of a single gene mutation; they may rather be the common consequence of variants in several genes. Second, they appear to result from the accumulation of deleterious variants; in fact 12 out of 13 patients had 3 or more risk alleles (a combination not found in any women of the control group). Third, the allelic variants reside in genes which do not belong to the group of genes classically associated with bone remodeling, and, consequently, the mechanisms explaining the association remain to be established.

The hedgehog family includes several proteins that influence skeletal biology, including Indian hedgehog and Sonic Hedgehog. They play an essential role in determining the shape and size of long bones. In fact, the hedhehog family of proteins is essential for skeletal patterning during development, as revealed by the limb skeletal malformations in hedgehog knock-out mice. HHAT encodes Hedgehog acyltransferase, an enzyme necessary for the palmitoylation of hedgehog proteins. An inadequate HHAT activity has been associated with reduced secretion and multimerization of hedgehog and developmental bone defects [17].

The protein encoded by ACKR3 (also known as CXCR7) was considered as an orphan receptor for many years, but now stromal cell-derived factor-1 (SDF1) has been identified as one of its ligands [18]. The interaction of

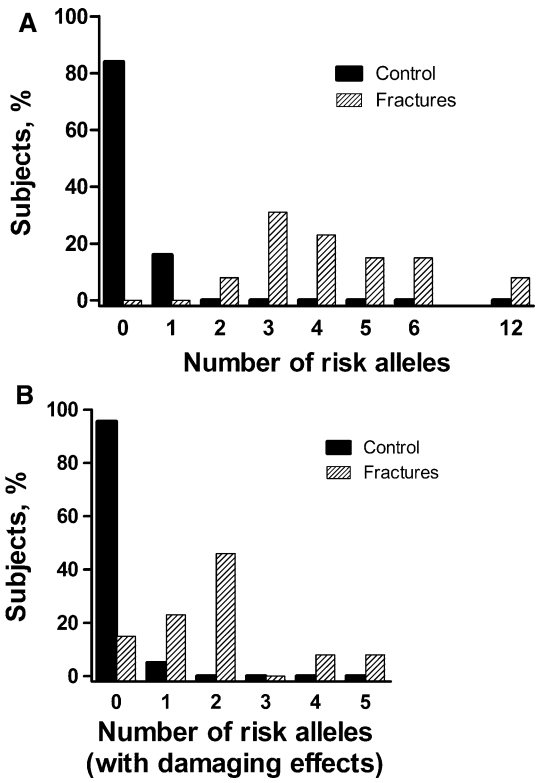

Fig. 1 Frequency distribution of the cumulative number of risk variants (those included in Table 1) in women with atypical fractures and in the control population (**a**). Analysis limited to those variants likely to have damaging effects on protein function (**b**)

CXCR7 with SDF1 modulates the activity of mesenchymal stem cells, precursors of osteoblasts and chondrocytes, and their response to a number of signals [19].

In view of these data, it is possible to speculate that the missense variants in HHAT and ACKR3 genes may influence the development, growth, and adaptation of the long bones of the lower extremities, which, in some way may predispose to individuals bearing the variant alleles to atypical fractures. In line with this "developmental" hypothesis, it is interesting to note that a particularly curved femoral shape appears to be associated with atypical fractures [20]. The common occurrence of bilateral and symmetric atypical fractures is also consistent with a role of lower limb geometry [21, 22]. Experimental studies may confirm this hypothesis in the future. It is interesting to note that although we and others have shown that polymorphisms of farnesyl diphosphate synthase (FDPS), the main target of aminobisphosphonates, are associated with the changes in bone mineral density in response to these drugs [23], we did not find evidence for association with atypical fractures. Nevertheless, this was not completely unexpected, for atypical fractures have been reported in patients treated with other anti-resorptive agents with a different mechanism of action [24, 25].

To our knowledge, this study represents the first attempt to determine the genetic factors involved in atypical femoral fractures. We used a genome-wide approach focused in coding non-synonymous variants, thus increasing the likelihood of finding functional variants associated with the disease. Along healthy women, we included patients with osteoporosis, but without atypical fractures, as the comparison group, in an attempt to avoid the potential risk of picking osteoporosis-related variants if women with atypical fractures were compared with healthy women. Given the small number of patients with atypical fractures, future replication in other cohorts will reinforce the biological importance of the risk alleles found in this study. Nevertheless, our results suggest a polygenic nature of atypical fractures. Hence, studies in other groups of patients will likely find other risk variants, missed in our study with a limited number of cases. Likewise, sequencing studies may discover additional variants not explored by the probes used in exon arrays.

In conclusion, we present a first attempt to explore the genetic architecture of atypical femoral fractures. Our study suggests that these fractures are polygenic and result from the accumulation of changes in the coding regions of several genes, some of which are related to skeletal development.

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Compliance with Ethical Standards

Conflict of interest Isabel Pérez-Núñez, José L. Pérez-Castrillón, María T. Zarrabeitia, Carmen García-Ibarbia, Laura Martínez-Calvo, José M. Olmos, Laisa S. Briongos, Javier Riancho, Victoria Camarero, Josep M. Muñoz Vives, Raquel Cruz and José A. Riancho declare that they have no conflicts of interest.

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